의학박사 학위논문

돼지-영장류 간 이종 심부표층각막이식에서 항 CD40 단클론항체의 유효성 및 안전성

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2017 년 7 월

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이 논문을 의학박사 학위논문으로 제출함

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김동현의 의학석사 학위논문을 인준함

2017 년 6 월

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ABSTRACT

Purpose: Corneal xenotransplantation is an effective solution for the shortage of human donor corneas, and the porcine cornea may be a suitable candidate for the donor cornea because of its optical similarity with humans. However, it is necessary to administer additional immunosuppressants to overcome antigenic differences. We aimed to investigate the feasibility of porcine corneas with anti-CD40 antibody-mediated co-stimulation blockade in a clinically applicable pig-to-nonhuman primate corneal xenotransplantation model.

Methods: Five Chinese rhesus macaques underwent deep-lamellar corneal transplantation using clinically acceptable sized (7.5 mm diameter) porcine corneal grafts. The anti-CD40 antibody was intravenously administered on a programmed schedule. Graft survival, central corneal thickness, and intraocular pressure were evaluated. Changes in effector and memory T cell subsets and anti-αGal and donor-specific antibodies were investigated in the

blood, and the changes in complement levels in the aqueous humor and blood were evaluated. Memory cell profiles in the anti-CD40 antibody-treated group were compared with those in from the anti-CD154 antibody-treated group or rejected controls presented in our previous report. The changes in anti-αGal, non-αGal, and donor-specific antibodies after 6 months were compared with baseline values.

Results: Anti-CD40 antibody-mediated co-stimulation blockade resulted in the successful survival of xenocorneal grafts (> 389, > 382, > 236, > 201, and > 61 days), with 80% reaching 6 months of survival. Injection of anti-CD40 antibody considerably reduced the infiltration of inflammatory cells into the grafts and significantly blocked the complement response in the aqueous humor ($p = 0.0159$, Mann-Whitney U test). Systemic expansion of effector memory T cells was abrogated in the anti-CD40 antibody-treated primates compared with those in the rejected controls ($p<0.05$, Mann-Whitney U test). The levels of anti-αGal, non-αGal, and donor-specific antibodies at 6 months

were not significantly increased compared with baseline levels ($p > 0.05$, Wilcoxon signed rank test).

Conclusion: An anti-CD40 antibody-mediated blockade appears to be effective immunosuppressive approach for porcine corneal deep anterior lamellar xenotransplantation in primates. In the future clinical trials of xenocorneal transplantation, the anti-CD40 antibody will be effective in immunosuppression.

Keywords: anti-CD40 antibody, cornea, deep anterior lamellar keratoplasty, non-human primates, xenotransplantation

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CONTENTS

LIST OF TABLES AND FIGURES

INTRODUCTION

Corneal blindness is one of the major causes in permanent visual loss world-widely, which can be only cured by corneal transplantation.[1] In several Asian countries including Korea, severe shortages of donor corneas have persisted because Confucian culture make it hard to obtain corneas from cadavers.[2-4] Additionally, aging of population and increase of cataract and corneal refractive surgery gradually reduce population pool which can present donor cornea in the long term. To overcome a world-wide shortage problem in donor cornea, the use of artificial cornea, bio-engineered regenerative cornea using induced pluripotent stem cells (iPS), and corneal xenotransplantation has been actively investigated.[2-4] However, possibility of tumorigenesis and difficulty in purifying specific cellular population exist in bio-engineered technique with iPS, and artificial cornea can cause severe tissue necrosis or immune reaction. Presently, corneal xenotransplantaion may be most feasible compared to iPS technology or artificial cornea because pig cornea is optically and physiologically comparable with human cornea, and pig can easily breed without major ethical problems.

The major hurdles of xenotransplantation coming into clinical trials are Galalpha1,3Galbeta1,4GlcNAc-R (αGal) epitope-mediated rejection and porcine endogenous retroviruses (PERVs)-mediated zoonosis.[5,6] Recent advances in xenotransplantation by development in a-1,3-galactosyltransferase gene knockout (GTKO) pigs and by development of genome-wide inactivation of PERVs using CRISPR-Cas9 takes us a step closer to commercially available xenografts and clinical trials along with the emerging evidence of the lack of transmission of PERVs in human studies.[7-9] Nevertheless, using GTKO pigs as a donor, optimized immune modulatory regimen should be still required as a critical step in xenotransplantation to achieve a successful outcome.[10] Recent long-term success of porcine corneal xenografts by anti-CD154 antibody (Ab)-mediated costimulation blockade in non-human primates (NHPs) created the possibility of clinical trials in corneal xenotransplantation.[11] However, anti-CD154 antibodies appear to greatly increase the incidence of thromboembolic complications and are impossible to use in human, despite a remarkable effect on transplantation.[12-15] Anti-CD40 antibody can be an alternative target in CD40/CD154 costimulation blockage

pathway. CD40 is expressed on B cells, macrophages, and dendritic cells, and CD8 T cells, however the thromboembolic complications with anti-CD154 therapy appear to be related with CD40-independent pathway.[16] Therefore, immunosuppression with anti-CD40 antibody may be potent and safe in xenocorneal transplantation.[16]

Accordingly, we investigated the effect of anti-CD40 Ab-mediated costimulation blockade on clinical efficacy using a pig-to-NHP deep lamellar-thickness corneal transplantation model with clinically applicable surgical procedures.

MATERIALS AND METHODS

All procedures used in this study conformed to the ARVO Statement Regarding the Use of Animals in Ophthalmic and Vision Research. The primate study protocol was approved by the Research Ethics Committee at Seoul National University Hospital [IACUC No. 13-0221-C1A0(1)].

Donors and recipients

Porcine corneas were obtained from genetically unmodified, pathogen-free SNU miniature wild type (WT) pigs that had been inbred in the Xenotransplantation Research Center at Seoul National University Hospital. Five Chinese rhesus macaques were used as recipients for deep anterior lamellar-thickness porcine corneal grafts (Table 1).

Orthotopic pig-to-rhesus deep anterior lamellar corneal transplantation

The donor corneoscleral buttons had been preserved in Optisol (Chiron Ophthalmics, Irvine, California) for 1~5 days before transplantation. Anterior

lamellae in donor cornea were incised 687.5- to 750-μm-thick with a 7.5-mm-diameter Barron vacuum trephine (Katena Products Inc., Denville, NJ) and artificial anterior chamber (Moria, Pennsylvania, USA). Deep-lamellar central portions of corneas were incised from recipients' eyes using 7.5-mm-diameter Hessburg-Barron vacuum trephines (Katena Products Inc., Denville, NJ) and the endothelial layer was separated from the full stromal layer using a big bubble technique as previous reported.[17,18] After removal of deep anterior lamellar recipient stromal layer, xenografts were placed in each recipient bed and secured with 16~20 interrupted 10-0 nylon sutures (Ethicon, Somerville, NJ). At the end of the surgical procedure, a contact lens (Acuvue® Oasys™, Johnson & Johnson, Jacksonville, FL) was inserted, and eyelids were closed with 6-0 black silk for 1 day.

Postoperative local and systemic immunosuppressive regimen

Postoperatively, all the recipients received the same medications. Levofloxacin 0.5% (Cravit®, Santen Pharmaceutical, Osaka, Japan) and prednisolone acetate 1% (Pred forte®, Allergan, Irvine, CA) were topically

administered once a day. Dexamethasone 1.5 mg/0.3 ml (JW Pharmaceutical, Seoul, Republic of Korea) was injected weekly subconjunctivally for up to 6 months and methylprednisolone (Solu-medrol®, Pfizer, New York, NY) was injected intramuscularly at an initial dose of 2 mg/kg/day, tapered over 5 weeks, and discontinued at a final dose of 0.25 mg/kg.[19] Intravenous immunoglobulin G (IVIG) and anti-CD 40 Ab were administered during the 6 months after transplantation. IVIG (1g/kg) was slowly administered on day 0 and 2 weeks post-transplantation. A mouse-rhesus chimeric monoclonal anti-CD40 (2C10R4, NIH Nonhuman Primate Reagent Resource) previously described,[20] was administered intravenously at a dose of 50~30mg/kg/day on day -1, day 1, postoperative day (POD) 4, POD 7, POD 10, and POD 14, once weekly until week 4, every 2 weeks until week 12, and then every 4 weeks until week 24 (15 times in total, 50 mg/kg/day in first 8 times and 30 mg/kg/day in the remaining 7 times), similar to the time schedules reported previously.[21]

Postoperative clinical evaluations

Grafts were evaluated by slit-lamp biomicroscopy, particularly for graft clarity, edema, and neovascularization. A graft rejection score on a scale of 0 to 12, was calculated based on the combined scores of graft clarity, edema, and neovascularization—each of which was scored on a scale of 0 to 4. The scoring system used was as follows[22, 23]: for clarity: 0, clear cornea; 1, slight haze; 2, increased haze but anterior chamber structures still clear; 3, advanced haze with difficult view of the anterior chamber; 4, opaque cornea without view of the anterior chamber; for edema: 0, no stromal or epithelial edema; 1, slight stromal thickness; 2, diffuse stromal edema; 3, diffuse stromal edema with microcystic edema of epithelium; 4, bullous keratopathy; for neovascularization: 0, no vascularization at graft-host junction (GHJ); 1, vascularization at GHJ in one quadrant only; 2, vascularization at GHJ in two quadrants only; 3, vascularization at GHJ in three quadrants only; 4, vascularization at GHJ in all four quadrants. Graft scores exceeding 6 and corneal clarity for 4 weeks was considered graft rejection. During the follow-up period, loosened, exposed, or new vessel-inducing sutures were selectively removed. Central corneal thickness (CCT) and intraocular pressure (IOP)

were measured using an ultrasonic pachymeter (Quantel Medical, Clermont-Ferrand, France) and a Tono-Pen (Medtronic Solan, Jacksonville, FL), respectively. The results are the mean values of 5 measurements. For visualization for living endothelial cells, enucleated eyeballs from the sacrificed recipients were evaluated using a SP-3000 specular microscope (Topcon Medical Laser Systems, Inc., Santa Clara, CA). The changes of endothelial cell density were compared between before and 6 months after transplantation in subjects in which data of endothelial cell density of donor were available before transplantation (n=3). For evaluation of corneal thickness and astigmatism, examinations using Orbscan IIz topography system (Bausch & Lomb, UT, USA) and anterior segment optical coherence tomography (Carl Zeiss Meditec, AG, Oberkochen, Germany) were performed in enucleated eyeballs.

Immunohistochemistry of xenografts

Corneas from sacrificed recipients were divided into two equal parts and each portion of the cornea was subjected to hematoxylin and eosin (H&E),

immunohistochemical and immunofluorescent staining as described previously.[11] Immunohistochemical staining of the grafts was performed using anti-CD68 Abs (Thermo Scientific, Runcorn, United Kingdom; 1:100) and Diaminobenzidine (DAB)-conjugated secondary antibody (Roche, Mannheim, Germany) for visualization. Immunofluorescence staining of the grafts was performed using (1) anti-CD3 Abs (Abcam, Cambridge, USA, 1:200) and goat anti rabbit IgG-FITC (SouthernBiotech, Birmingham, USA, 1:500) as well as anti-CD8 Abs (Abcam, Cambridge, USA; 1:150) and goat anti mouse IgG-Cy3 (Millipore, California, USA, 1:500) for $CD3^+CD8^+$ T cells; (2) anti-CD3 Abs (Abcam, Cambridge, USA, 1:200), goat anti rabbit IgG-Cy3 (Millipore, California, USA, 1:200) and anti-CD4-alexa fluor 488 conjugated Abs (Novusbio, Littleton, USA; 1:50) for $CD3^+CD4^+$ T cells; and (3) anti-CD3 Abs (Abcam, Cambridge, USA, 1:200) and goat anti rabbit IgG-FITC (SouthernBiotech, Birmingham, USA, 1:500) as well as anti-CD20 Abs (eBioscience, San Diego, USA; 1:100) and goat anti mouse IgG-Cy3 (Millipore, California, USA, 1:500) for $CD3^-CD20^+$ B cells. Counter nuclear staining was done using DAPI (4',6-diamidino-2-phenylindole). To

investigate the distribution of αGal epitopes, the Griffonia simplifolia I isolectin B4 (GSIB4; Molecular Probes, Eugene, OR) conjugated with Alexa fluor 488 (I-21411; Molecular Probes) was reacted with the corneal tissue as described previously.[11] To confirm the deposition of IgG Abs in the corneal tissues, fluorescein isothiocyanate (FITC) polyclonal goat anti-monkey IgG (Fc specific) Abs (AP31438FC-N; 1:20, Acris Antibodies Inc., San Diego, CA) were used, per the manufacturer's protocol. To confirm the deposition of complement C3c in the corneal tissues, polyclonal rabbit anti-human C3c Abs (A0062; 1:100, DAKO) were used as a primary Ab and Alexa Fluor 488 goat anti-rabbit IgG Abs (A11304; 1:500, Life technologies, Grand Island, NY) were used as the secondary Ab. The nuclei of cells were then counterstained with Hoechst33342 and the distribution of IgG Ab and complement C3c was investigated with Venoz AHBT3/Q fluorescent microscopy (Olympus, Tokyo, Japan). Histologic findings in anti-CD40 treated primates were compared with rejected controls (n=2, Table 1) that were given topical and systemic steroid after keratoplasty, using a previous study as the reference.[19]

Memory T cell assay

Postoperative changes in memory T-cell sub-populations (CD28+CD95+ central memory and CD28-CD95+ effector memory) were evaluated by flow cytometry analysis of whole blood and draining lymph nodes as previously mentioned.[11] Data were acquired using a FACSCanto flow cytometer (Becton-Dickinson, Mountain View, CA) and data analysis was carried out using FlowJo software (Tree Star, Ashland, OR). The changes of memory T cell expansion of the blood over time in anti-CD40 Ab-treated primates (n=5) were compared to those in anti-CD154 Ab-treated primates (n=4) with porcine corneal grafts which survived successfully as previous described (as a reference) to confirm whether the T cell responses are effectively controlled in tolerable range.[11,19] In addition, mean percentages and numbers of effector or central memory T cells in draining lymph nodes were compared between anti-CD40 Abs-treated primates (n=4) and rejected controls (n=2, Table 1) that were given topical and systemic steroid after keratoplasty, using a previous study as the reference.[11,19] The lymph nodes of anti-CD40 treated primates were collected after 6 months and those of the rejected controls in

which lymph nodes had been available were collected after 1months on sacrifice.

Anti-αGal and anti-non-αGal antibodies assay

Plasma levels of anti-αGal IgG and IgM Abs were measured by ELISA as described previously.[24,25] In brief, mean absorbance of anti-αGal Abs was expressed as artificial units (AU)/mL, and was compared with that of a calibrator. As a calibrator, serial dilutions of a selected rhesus monkey plasma were designated as 2,809AU/mL of anti-αGal IgG and 5,610AU/mL of anti-αGal IgM. Anti-αGal IgG Ab or anti-non-αGal IgG Ab binding responses were assessed by flow cytometry using a GTKO porcine endothelial cell lines (PEC69), and for supplemental purposes, WT porcine endothelial cells (MPN) incubated with 1/10 diluted plasma samples.[24] The level of antibody binding was determined as the net mean fluorescence intensity (nMFI). nMFI values were calculated by subtracting MFI values of the porcine plasma (negative control) from MFI values of the sample. The plasma concentration or MFI

were measured every 2 weeks up to 4 months, and thereafter every 1 month until sacrifice. Mean MFI levels and plasma concentration of anti-αGal IgG and IgM Abs or anti-non-αGal IgG Ab at 6 months were compared with those prior to treatment (n=4).

Donor pig-specific antibodies assay

Plasma concentrations of donor pig-specific IgM/IgG Abs were determined by the flow cross-match technique as described previously using donor peripheral blood mononuclear cells (PBMCs) as targeting cells.[11] Concentrations of donor pig-specific Abs were semi-quantitatively expressed as the MFI. MFIs of donor serum were used as negative controls and net MFI (nMFI) values were calculated by subtracting donor MFI values from respective sample MFI values. The plasma concentration or MFI were measured every 2 weeks up to 4 months, and thereafter every 1 month until sacrifice. Mean MFI levels of donor-specific IgG and IgM Abs at 6 months were compared with those prior to treatment (n=4).

Complement assay in aqueous humor

Concentration of C3a in the aqueous humor was evaluated by commercial ELISA kits (BD OptEIA™ Human C3a ELISA Kit; BD Biosciences, San Diego, CA) according to the manufacturer's protocols prior to transplantation and 1 months, 3 months and 6 months after transplantation. The changes of complement in anti-CD40 Abs treated primates (n=5) at 1 month were compared to those in controls with rejected grafts (n=4, Table 1) that were given the topical and systemic steroid in previous study or topical and systemic steroid combined with tacrolimus and IVIG after keratoplasty as a reference.[19] The changes of complement in anti-CD40 Abs treated primates at 6 month were also compared with those prior to surgery (n=4).

Safety monitoring

Safety monitoring in anti-CD40 Abs treated primates was performed because potent immunosuppression of anti-CD40 Abs may cause several systemic disorders. Weight, body temperature, complete blood discount (CBC), and blood chemistry panel (liver, renal, and electrolyte panel) were measured

every 2 weeks until week 12, and then every 4 weeks thereafter. Reactivation of rhesus cytomegalovirus (CMV) was also periodically checked until sacrifice. The quantification of RhCMV was determined by real-time PCR using forward and reverse primers, 50- ACAGAGGCCAGTGGGATGTC-30 and 50-CCCTGATGATGGGCATAGATAAG-30 and probe 50 FAM-CCAGGCACATTCTCTGGGAGCACAC-30 TAMRA (Bioneer Co., DaeJeon, Korea). Their sequences are located in the highly conserved RhCMV 156 region and specific to RhCMV IE2.[26] Anti-RhCMV antibody was measured using immunofluorescence assay.

Statistical analyses

GraphPad Software (GraphPad Prism, Inc., La Jolla, CA, USA) was used for statistical tests. Mean values of C3a concentration in the aqueous humour were compared between anti-CD40 Abs-treated primates and rejected controls using the Mann-Whitney U test. Mean percentages or numbers of memory T cells in draining lymph nodes or blood samples were also compared between anti-CD40 Abs-treated primates and rejected controls or between anti-CD40

Abs-treated primates using the Mann-Whitney U test. The changes in anti-αGal IgM and IgG Abs or donor-specific antibodies, the changes in complement and corneal endothelial cell density were compared between the pre-operative state and 6 months after surgery using the Wilcoxon signed rank test. Data are presented as mean ± standard error (SE) except demographics of donors and recipients. Statistical significance was accepted for p values <0.05.

RESULTS

Clinical outcomes

All recipients had transparent xenocorneal grafts longer than 6 months after the transplantation except one that died due to anesthetic accident at 2 months (A clear graft was evident until death). In addition, two recipients (anti-CD40 1 and anti-CD40 2) showed successful survival longer than a year without any signs of rejection (Figure 1). No corneal neovascularization was observed in all primates.

The MST of the anti-CD40 Ab group ((n=5); >389, >382, >236, >201, and >61 days; Table 1) could not to be defined because all xenocorneal grafts of five recipients had survived throughout the whole study. The international guideline for a clinical trial of xenocorneal transplantation demands at least 6

months-survival of 5 in a consecutive 8 NHPs to prove an efficacy as a pre-clinical data.[24] For the welfare and animal-rights of NHPs, we tried to use minimal quantity of NHPs as possible as we could. Unfortunately, a primate had been dead during the anesthetic recovery at 2 months because the neck was accidentally flexed. However, we did not use additional NHPs, because in 5 consecutive NHPs, we did not experience any rejection at all. Although the graft had been still survived after 6 months in remaining NHPs, we had to sacrifice them due to limitation of the funding. Rejection scores at sacrifice were 0.20±0.44 and 8.25±0.96 in the anti-CD40 antibodies and control group respectively. ($p=0.010$, Mann-Whitney U test)

The CCT was well maintained under 600μm throughout the follow-up period and IOPs were not increased (Figure 1C), and the density and shape of endothelial cells were well maintained in all recipients without significant reduction ($p>0.05$, Wilcoxon signed rank test, Figures 2). Anterior segment optical coherence tomographic images of the enucleated eyes showed a well-adapted donor-recipient junction and topography showed tolerable range of the refractive diopter and astigmatism (Figure 2). Table 2 showed summarized

results of clinical examinations in enucleated eyeballs of anti-CD40 antibodies treated primates at sacrifice.

Neither ocular nor systemic adverse events including side effects of anti-CD40 Ab or cytomegalovirus (CMV) infection due to long-term immunosuppressive therapy were observed in any recipients (Figure 3 and 4).

Cellular infiltration into the grafted porcine cornea

In H&E stains, all recipients with anti-CD40 Abs treatment showed clear grafts with a few inflammatory cells confined in the donor-recipient junction (Figure 5A), while rejected control showed severe edema accompanying by abundant inflammatory cellular infiltration (Figure 5B). In immunohistochemical and immunofluorescent staining, CD3+CD4+ T cells, CD3+CD8+ T cells, CD3-CD20+ B cells and CD68+ macrophages were scarcely found in the surviving grafts (Figure 5A and Figure 6A; upper panel), while infiltration of those cells was abundantly observed in grafts of the rejected control, especially in graft-host junction (Figures 5B and 6B; lower panel).

Plasma donor pig-specific antibody assay and IgG deposition in porcine corneal grafts

The level of anti-donor specific IgM and IgG Ab were not changed throughout the follow-up period (Figures 7A, B and E) and differences were not statistically significant between the baseline and 6 month post-operative time point ($p>0.05$, Wilcoxon signed rank test). Transient peak of IgG Abs in early post-operative period may be caused by non-specific bindings of previously existing antibodies in IVIG (Figure 8A). Immunofluorescent staining showed IgG deposits were barely observed in the graft with anti-CD40 Ab-treatment (Figure 7C), while deposits were densely found in the graft in the rejected control (Figure 7D).

Plasma anti-Gal antibody assay and αGal expression in porcine corneal grafts

The level of anti-αGal IgM Ab was not increased throughout the study period (Figure 9A, E). The level of anti-αGal IgG Ab showed subtle increase only in early postoperative period due to initial treatment of IVIG, and this peak had

no correlation with donor-specific responses (Figure 8). Thereafter, there was no increase of the level of anti αGal IgG Ab through the follow-up, based on baseline verses 6 month values (Figure 9B and E; $p>0.05$, Wilcoxon signed rank test). There were no significant changes in MFI values in the plasma samples against wild type porcine endothelial cells (PEC; Figure 9C, F; $p>0.05$, Wilcoxon signed rank test) or against GTKO PEC (Figure 9D) at 6 months compared to baseline (Figure 9F; $p>0.05$, Wilcoxon signed rank test), suggesting no significant induction of anti-αGal or anti-non-αGal antibodies in the recipients. In immunofluorescent staining, anti-CD40 Ab-treated primates showed few αGal-expressing cells in the graft (Figure 9G), while the rejected control showed dense αGal-expressing cells (Figure 9H).

Aqueous complement C3a assay and complement C3c deposition in porcine corneal grafts

There was no clinically relevant increase in C3a concentration in aqueous humor at 6 months compared with baseline level (Figure 10A, E; $p>0.05$, Wilcoxon signed rank test). At 4 weeks after transplantation, the mean

aqueous C3a concentration was significantly lower in anti-CD40 Abs treated primates than that in rejected controls (p=0.0159, Mann-Whitney U test; Figure 10B). The grafts of the anti-CD40 Ab-treated primates did not show a deposition of C3c in the graft (Figure 10C), while graft of the rejected control showed pronounced deposition of C3c in the graft (Figure 10D).

Memory T cell changes in whole blood and draining lymph nodes

The level of interferon-gamma (IFNγ) secreting CD4+ and CD8+ T cells, effector memory (CD28-CD95+) and central memory (CD28+CD95+) T cells were not higher than those in preoperative states throughout the follow-up (Figure 11A-F). When we compared the IFNγ+ T cells or memory and effector CD4+ and CD8+ T cells between the baseline and 6 months, there was no statistical significant increase over time in anti-CD40 treated primates (Figure 11G-L; $p>0.05$, Wilcoxon signed rank test). When we compared concentrations of those memory T cells at baseline, 1 month, and 6 months between anti-CD40 treated and anti-CD154 treated primates which showed

effective suppression of the clinical rejection, the concentration of CD8+ central memory T cells was significantly lower in anti-CD40 treated primates at 1 month (Figure 11L; p=0.0317, Mann-Whitney U test). The other memory T cell concentrations tend to be lower without statistical significance, (Figure 11G-K; p>0.05, Mann-Whitney U test). In the draining lymph nodes, mean percentage of central memory (CD28+CD95+) CD4+ T cells was lower in anti-CD40 Ab-treated primates than in rejected controls with a marginal significance (p=0.082, Mann-Whitney U test; Figure 12C). The number of effector memory (CD28-CD95+) CD4+ and CD8+ T cells were lower in anti-CD40 Ab-treated primates than in rejected controls (p=0.055, p=0.032, Mann-Whitney U test; Figure 12E). The changes of IFNγ+ CD4+ and IFNγ+ CD8+ T cells tended to decrease with treatment, but were not statistically significant (Figure 12A, D).

Table 1. Demographics of donors and recipients and graft survival with anti-CD40 Ab-primates. Demographics of anti-CD40 Ab-primates are presented. Rejected controls given topical and systemic steroid or steroid with tacrolimus, are included as a reference for use in comparison of memory T cell, antibody or complement responses.

	Recipients			Donors		Immuno-suppressive Regimen	Graft Survival (days)	Rejection score (at sacrifice)
	Age (months)	Body Weight (kg)	ABO type	Age (months)	ABO type			
Anti-CD40 1	140	6.32	B	28	A	Topical prednisolone+	>389	0
Anti-CD40 2	74	7.12	A	28	A	Subconjunctival	>382	0
Anti-CD40 3	56	5.02	AB	30	A	Dexamethasone+ Intramuscular	>236	0
Anti-CD40 4	57	5.60	B	28	A	Methylprednisolone+ IVIG+	>201	1
Anti-CD40 5	57	4.80	B	28	A	Intravenous anti-CD40 Ab	>61	0
Mean ± SD	56.7±0.58	5.78±0.96		28.40±0.89			MST : Undefined	0.20±0.44

Control 1*	48	5.48	A	37	A	Topical prednisolone+ Subconjunctival Dexamethasone+ Intramuscular Methylprednisolone	28	8
Control 2*	54	4.90	AB	35	A		29	9
Control 3*	137	4.94	B	31	A		21	9
Control 4	57	4.2	AB	42	A	Topical prednisolone+ Subconjunctival Dexamethasone+ Intramuscular Methylprednisolone+ IVIG+Tacrolimus Intramuscular Tacrolimus	32	7
Mean ± SD	74.0±42.2	4.88±0.45		36.25±3.96			MST : 28.5	8.25±0.96

SD: standard deviation, MST: mean survival time

* were adopted from previous study.[11]

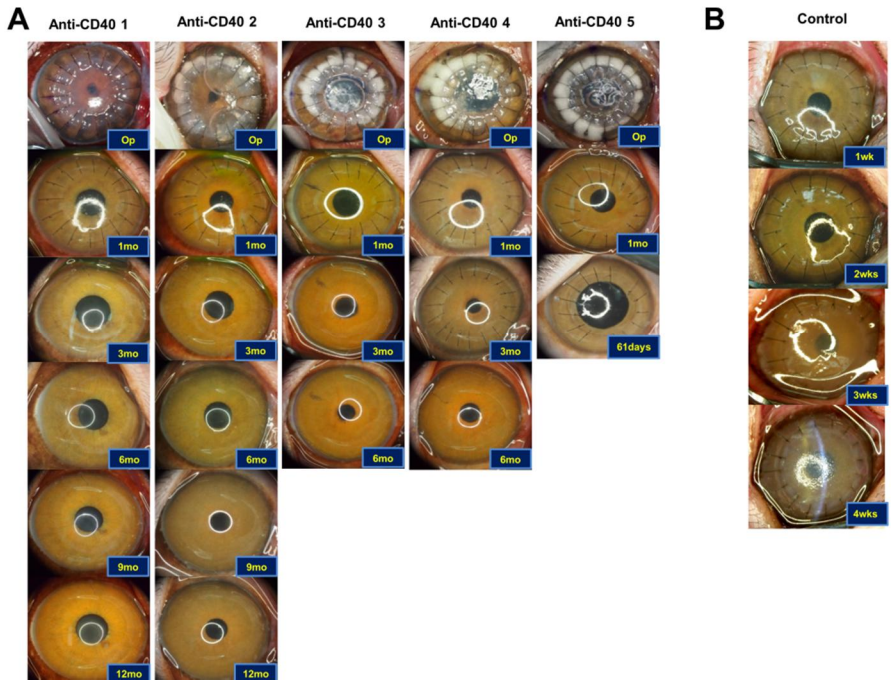
Table 2. Clinical examinations of enucleated eyeballs in anti-CD40 Abs treated primates at sacrifice.

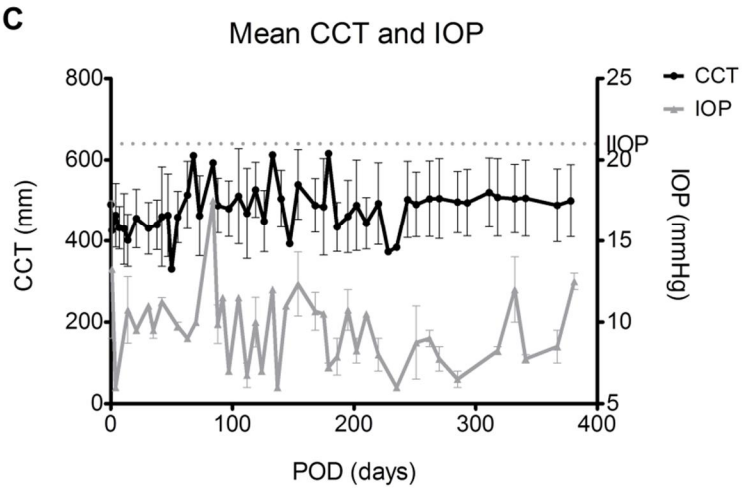
	ECD (cells/mm^2)	CV	HEX	Sim K max (D)	Sim K min (D)	ACD (mm)	CCT (μm)
Anti-CD40 1	3012	23	60	57.4	52.0	3.46	642
Anti-CD40 2	3472	35	65	56.6	50.5	2.89	442
Anti-CD40 3	2257	44	52	56.6	52.0	3.80	401
Anti-CD40 4	3175	38	53	58.9	51.9	2.92	630
Anti-CD40 5	3509	26	69	50.2	47.0	2.88	383
Mean ± SD	3085±507	33.2±8.6	59.8±7.4	55.9±3.3	50.7±2.2	3.19±0.42	499±126

ECD: corneal endothelial cell density; CV: coefficient of varition; HEX: hexagonality; Sim K max: maximum simulated keratometric value; Sim K min: minimum simulated keratometric value; ACD: anterior chamber depth; CCT: central corneal thickness; SD: standard deviation

Figure 1. Representative photographs of grafted eyes showing clinical courses, and changes of central corneal thickness and intraocular pressure in deep anterior lamellar porcine keratoplasty in nonhuman primates.

(A) All grafts in the anti-CD40 Abs treated primates survived at least 6 months except one (Anti-CD40 5) which accidently died due to anesthetic event at POD 61. The primate also showed clear graft until death. Two of the grafted primates showed more than 1year survival. (B) Representative photos of the control showing rejection within 4 weeks. (C) Central corneal thickness was well maintained under 600μm and increase of intraocular pressure was not observed during the follow-up in the anti-CD40 Abs treated primates.

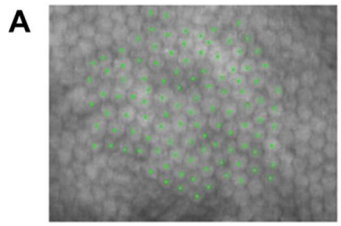


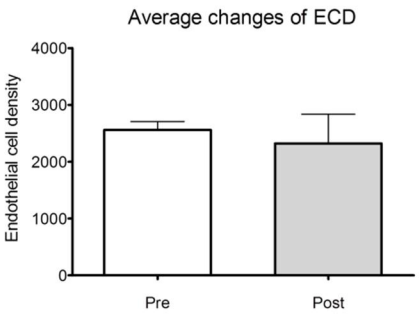


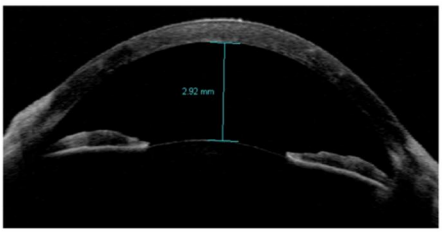
CCT: central corneal thickness; IOP: intraocular pressure

Figure 2. Representative of photographs of endothelial cell density (ECD) and refractive power in grafted eyes of anti-CD40 Abs treated primates at POD 201 and average data of ECDs in the surviving grafts.

(A) Endothelial cell density (ECD; CD) was well maintained showing 3401/mm^2, accompanying by normal range of coefficient of varition (CV; 26) and hexagonality (HEX; 72). (B) Average changes of ECD in the anti-CD40 Abs treated primates after sacrifice with at least 6 months-survival were not significantly different compared to basal levels (mean ± standard error; $p>0.05$, Wilcoxon signed rank test). The primates in which both pre- and post-operative ECD had been available were only included (n=3). (C) Anterior segment optical coherent tomography demonstrated well approximated wound and normal depth of anterior chamber. (2.92mm) (D) Topography showed maximum and minimum simulated K values of 58.9 and 51.9 diopter respectively.


A
CD= 3401
HEX= 72
CV= 26
AVE= 294
NUM= 74
AREA= 21721


B
Average changes of ECD
Endothelial cell density
4000
3000
2000
1000
0
Pre
Post


C
2.92 mm

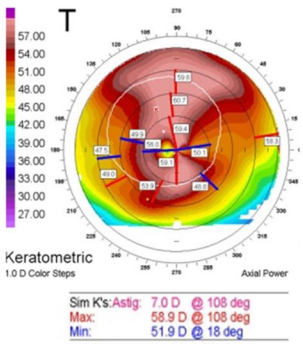

D
T
57.00
54.00
51.00
48.00
45.00
42.00
39.00
36.00
33.00
30.00
27.00
Keratometric
1.0 D Color Steps
Axial Power
Sim K's: Astig: 7.0 D @ 108 deg
Max: 58.9 D @ 108 deg
Min: 51.9 D @ 18 deg

Figure 3. Monitoring of rhesus cytomegalovirus in peripheral blood of the anti-CD40 Abs treated primates after corneal xenotransplantation. The copy numbers of Rhesus CMV (RhCMV, black line) DNA in plasma were determined by quantitative real-time PCR, and the antibody was titrated by immunofluorescence assay. Incidental increase of RhCMV copy number in early postoperative period was observed in anti-CD 40 treated primate 1 (anti-CD40 1, A), followed by subsequent increase of anti-RhCMV antibody responses, resulting in decrease of the copy number during the follow-up. Clinical signs of viremia were never observed. In all the other primates (B-D), anti-RhCMV antibody responses (gray dot line) consistently increased upon increase of the copy number of RhCMV, resulting in no clinically relevant increase of the copy number. It suggests that an intact humoral immune response to CMV reactivation.

CMV: cytomegalovirus; PCR: polymerase chain reaction.

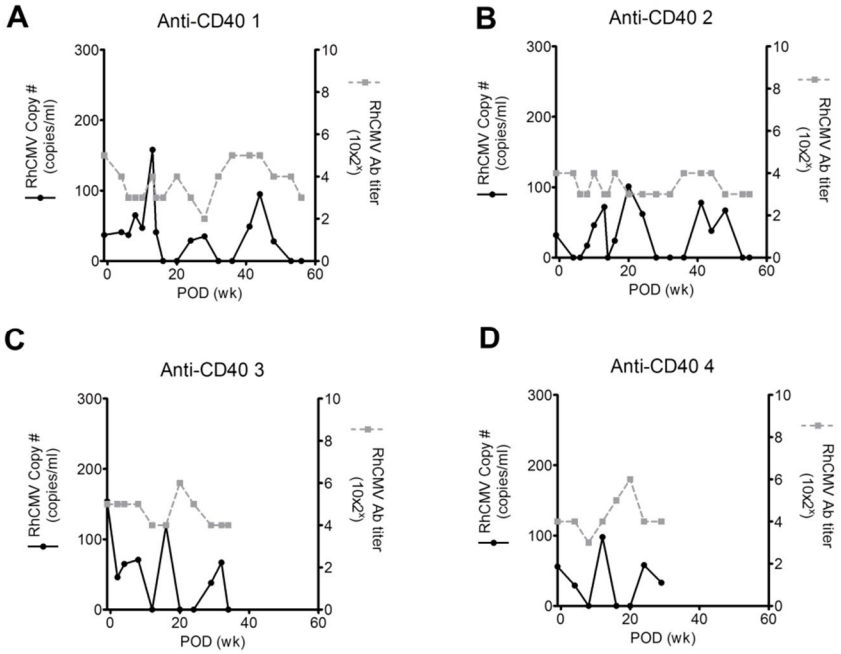

A
Anti-CD40 1
RhCMV Copy # (copies/ml)
RhCMV Ab titer (10x2^n)
POD (wk)
B
Anti-CD40 2
RhCMV Copy # (copies/ml)
RhCMV Ab titer (10x2^n)
POD (wk)
C
Anti-CD40 3
RhCMV Copy # (copies/ml)
RhCMV Ab titer (10x2^n)
POD (wk)
D
Anti-CD40 4
RhCMV Copy # (copies/ml)
RhCMV Ab titer (10x2^n)
POD (wk)

Figure 4. Safety monitoring in the anti-CD40 Abs treated primates.

No clinically significant changes were observed in weight, body temperature, CBC, renal, and liver panel.

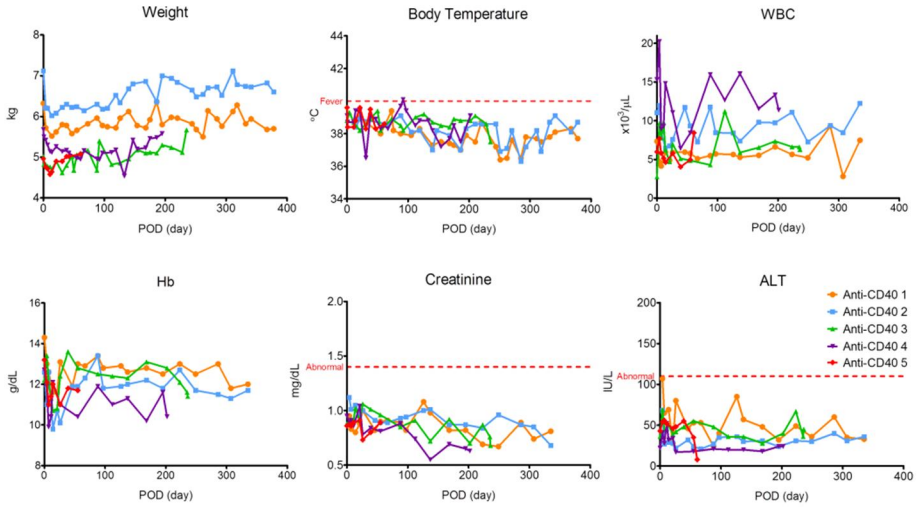
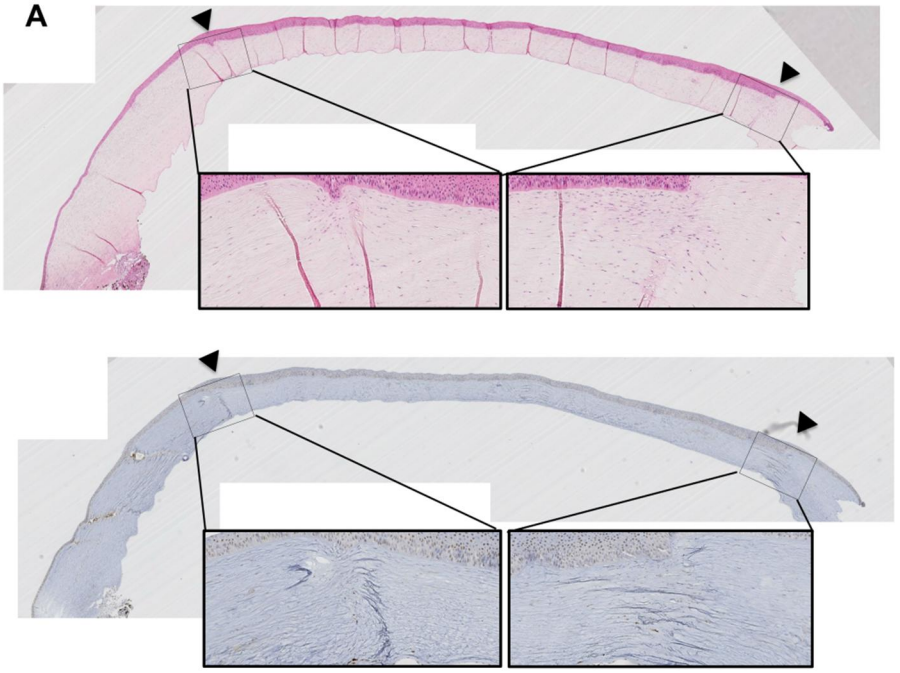


Figure 5. Representative findings in haematoxylin & eosin (H&E) and immunohistochemical staining (IHC) of porcine corneal grafts in the anti-CD40 Ab-treated primate and rejected control.

(A) Staining of a surviving graft in the anti-CD40 Abs treated primates showed less inflammatory cell infiltration without any graft edema (Top; H&E, bottom; IHC using anti-CD68 Ab; arrowheads indicate donor-recipient junction; X40 and X200). (B) Staining of a graft in the rejected control without anti-CD40 Ab treatment showed severe edema and inflammatory cell infiltration around the donor-recipient junction (Top; H&E, bottom; IHC using anti-CD68 Ab; arrowheads indicate the donor-recipient junction; magnification X40 and X200).


A

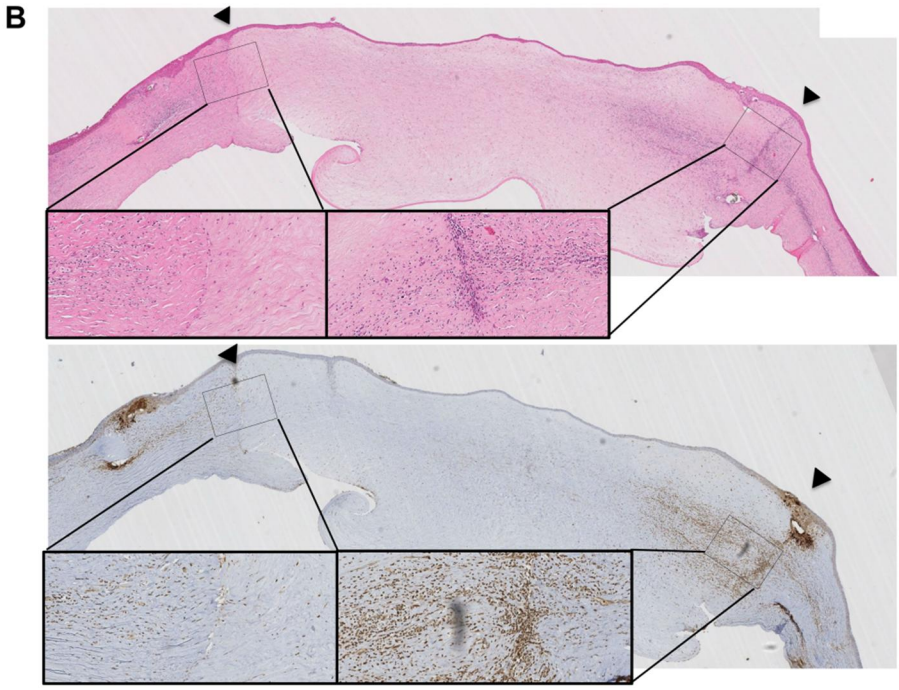

B

Figure 6. Immunofluorescent staining for inflammatory cells infiltrating into the porcine corneal graft in the anti-CD40 Ab-treated primate and rejected control.

A surviving graft in an anti-CD40 Ab-treated primate (A; upper panel, anti-CD40 Ab-treated primate, magnification x200) showed few cells, while a rejected graft (B; lower panel, rejected control; magnification x200) showed significant infiltration of CD3+CD4+ (CD3;red/ CD4;green double staining) and CD3+CD8+ (CD3; green/ CD8; red double staining) T cells as well as CD3-CD20+ (CD3; green /CD20; red) B cells (white arrows).

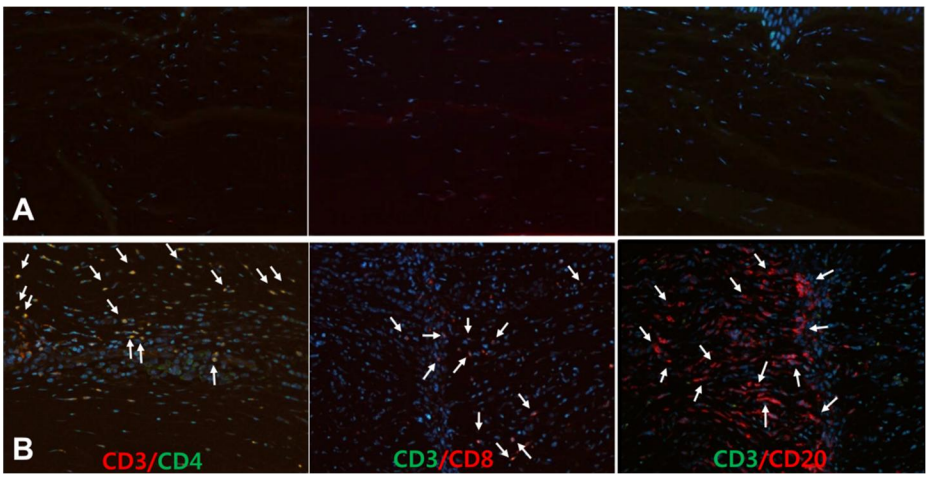


Figure 7. Changes in plasma donor pig-specific antibodies and IgG deposition in porcine corneal grafts with anti-CD40 Ab-treated primates.

Few changes were observed in donor pig-specific IgM (A) and IgG (B) antibodies in anti-CD40 Ab-treated primates during the follow-up. Initial peak of anti-IgG in early postoperative days was probably caused by intravenous immunoglobulin treatment. Anti-CD40 Ab-treated primate showed little IgG deposition in the stroma (C), while A rejected control showed dense IgG deposition mainly in the stroma and epithelium (D) (X100). Rejected graft showed relatively thin epithelial layers. Dash lines and white arrows indicate epidermal-dermal junction. (E) Compared with basal levels, the MFI level of donor pig-specific IgM and IgG antibodies was not significantly high at 6 months ($p>0.05$, Wilcoxon signed rank test; $n=4$). “Pre-op” indicates pre-operative baseline data, and “6 mo” indicates data at 6 months post-transplant.

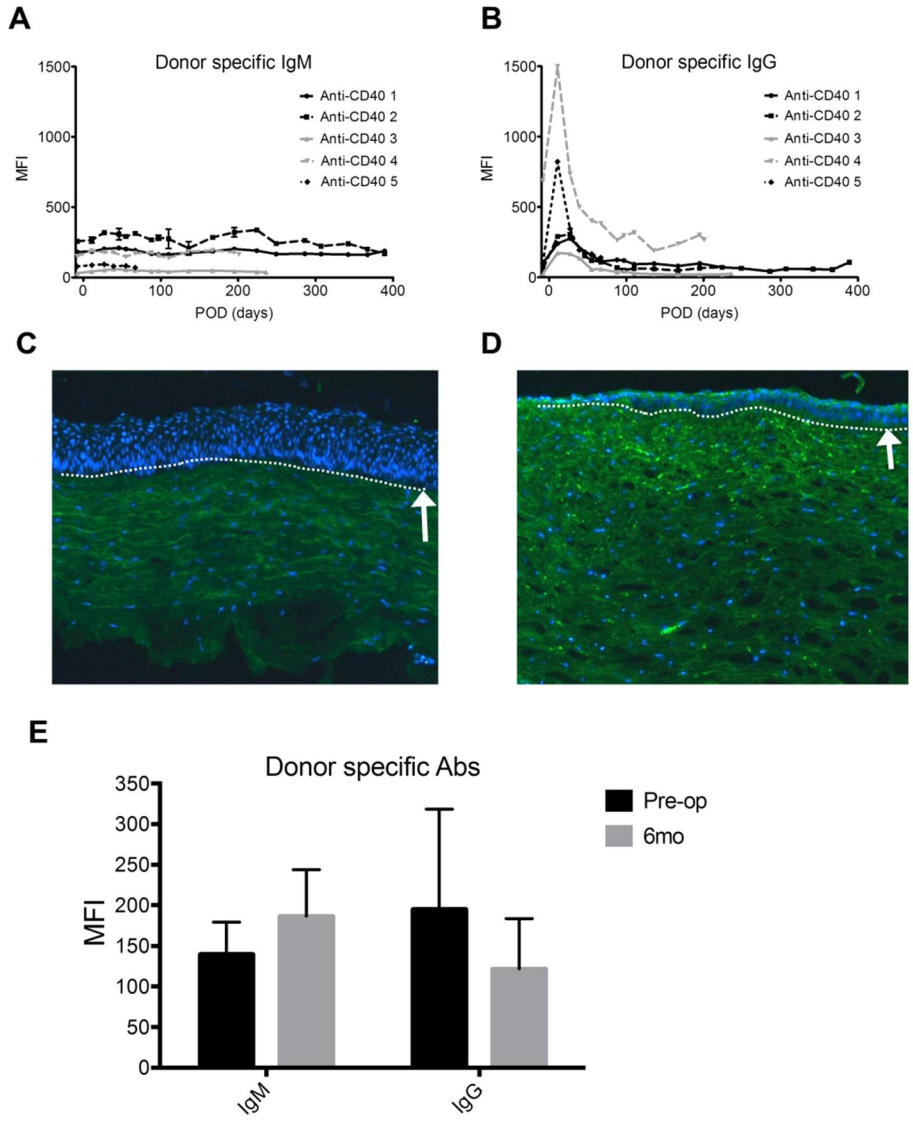

A
Donor specific IgM
MFI
1500
1000
500
0
0
100
200
300
400
POD (days)
Anti-CD40 1
Anti-CD40 2
Anti-CD40 3
Anti-CD40 4
Anti-CD40 5
B
Donor specific IgG
MFI
1500
1000
500
0
0
100
200
300
400
POD (days)
Anti-CD40 1
Anti-CD40 2
Anti-CD40 3
Anti-CD40 4
Anti-CD40 5
C
D
E
Donor specific Abs
MFI
350
300
250
200
150
100
50
0
IgM
IgG
Pre-op
6mo

Figure 8. The conpounding effects of IVIG administration *in vivo* or *in vitro* on donor pig-specific and anti-αGal antibody assays.

(A) The *in vivo* IgG binding responses to 3rd party PBMCs were assessed over time by flow cytometry using plasma of anti-CD40 Ab-treated primates (n=5) given IVIG infusion on day 0 and 2 weeks post-transplantation. Non-specific IgG binding against 3rd party PBMCs was observed during the early post-operative period, possibly due to infusion of IVIG that may already contain IgG Abs against porcine PBMCs. Data are presented as mean ±standard errors from 5 subjects. (B) The *in vitro* IgG binding responses against porcine PBMCs were assessed by flow cytometry using plasma of a naïve primate when mixed with IVIG agents from different lot numbers (#1, #2). Non-specific IgG binding against porcine PBMCs was also observed in a dose-dependent manner after addition of IVIG into the plasma sample. (C) The levels of anti-αGal IgG Abs were measured by ELISA in IVIG agents from different lot numbers (#1, #2) and in plasma of a naïve primate mixed with these IVIG lots. A detectable amount of basal anti-αGal IgG was found to

exist in the IVIG formulations.The addition of IVIG into the naïve monkey plasma elevated the level of anti-αGal IgG in a dose-dependent manner. (PBMCs=peripheral blood mononuclear cells, MP = monkey plasma and HSA = human serum albumin, w/=with)

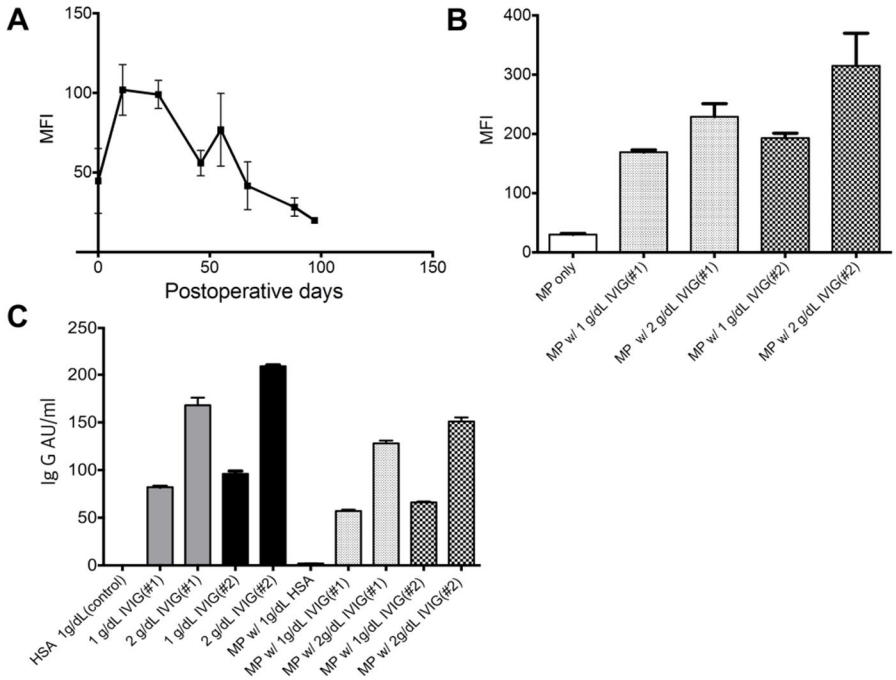


Figure 9. Changes in plasma anti-αGal antibodies or anti-non αGal antibodies and αGal expression in porcine corneal grafts with anti-CD40 Ab-treated primates.

No significant changes were observed in IgM (A) and IgG (B) anti-αGal antibodies during the follow-up period (E; baseline vs 6 months, $p>0.05$, Wilcoxon signed rank test; n=4). "Pre-op" indicates pre-operative baseline data, and "6 mo" indicates data at 6 months post-transplant. There were no significant changes in the MFI values of plasma samples against wild-type porcine endothelial cells (PEC; C) or against GTKO PEC (D) during the follow-up period compared to the pre-transplantation level (F; baseline vs 6 months, $p>0.05$, Wilcoxon signed rank test), suggesting no significant induction of anti-αGal or anti-non-αGal antibodies in the recipients. Anti-CD40 Abs treated primate showed few αGal expressions in the stroma (G), while the rejected control showed dense αGal expression (H) (X100). Dash lines and white arrows indicate epidermal-dermal junction.

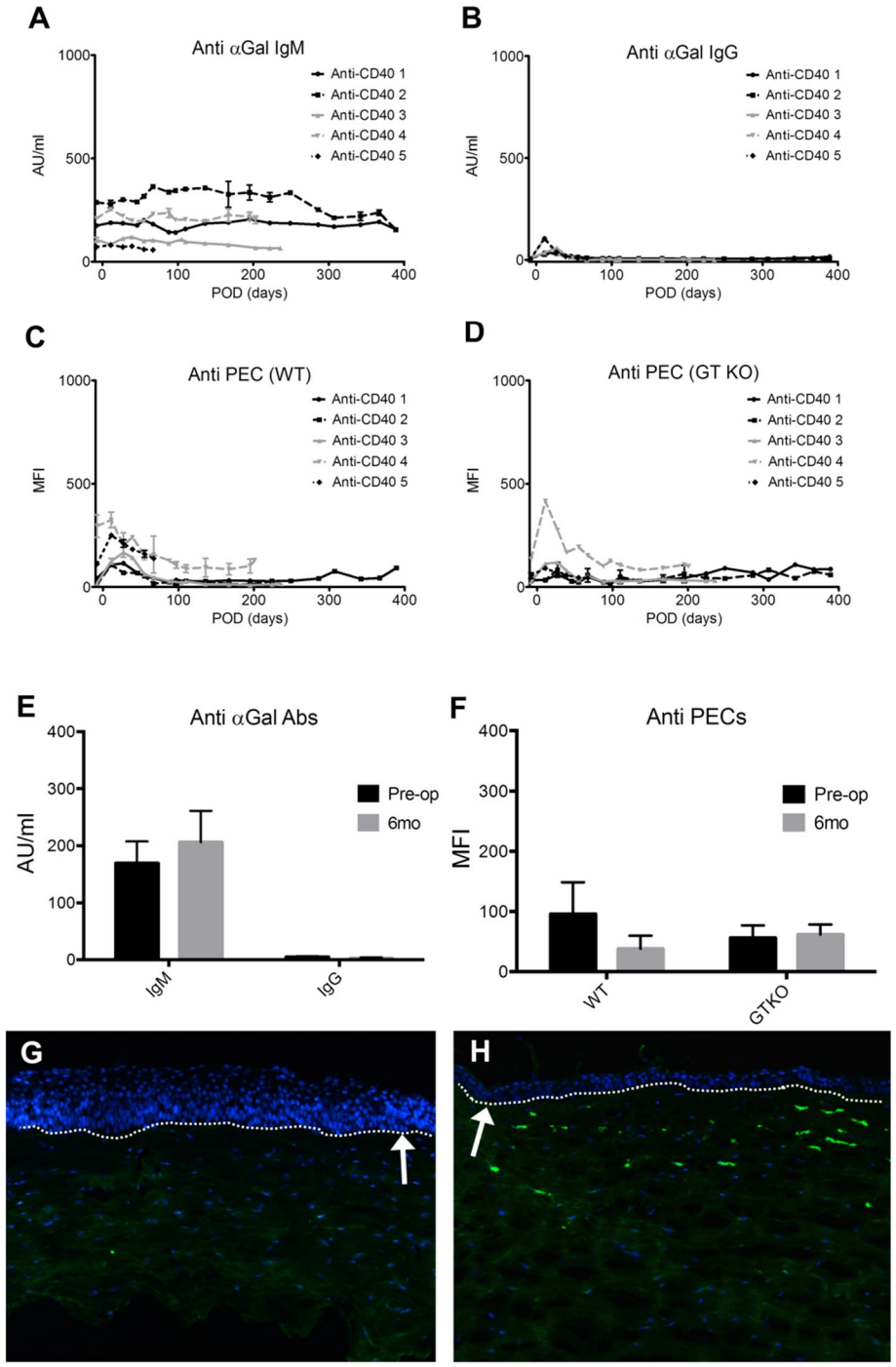

A
Anti αGal IgM
AU/ml
1000
500
0
0
100
200
300
400
POD (days)
Anti-CD40 1
Anti-CD40 2
Anti-CD40 3
Anti-CD40 4
Anti-CD40 5
B
Anti αGal IgG
AU/ml
1000
500
0
0
100
200
300
400
POD (days)
Anti-CD40 1
Anti-CD40 2
Anti-CD40 3
Anti-CD40 4
Anti-CD40 5
C
Anti PEC (WT)
MFI
1000
500
0
0
100
200
300
400
POD (days)
Anti-CD40 1
Anti-CD40 2
Anti-CD40 3
Anti-CD40 4
Anti-CD40 5
D
Anti PEC (GT KO)
MFI
1000
500
0
0
100
200
300
400
POD (days)
Anti-CD40 1
Anti-CD40 2
Anti-CD40 3
Anti-CD40 4
Anti-CD40 5
E
Anti αGal Abs
AU/ml
400
300
200
100
0
Pre-op
6mo
IgM
IgG
F
Anti PECs
MFI
400
300
200
100
0
Pre-op
6mo
WT
GTKO
G
H

Figure 10. Changes in concentration of aqueous humor C3a and C3c deposition in porcine corneal grafts in the anti-CD40 Ab-treated primate and rejected control.

(A, E) C3a concentration was not increased in the aqueous humor at 6 months compared to baseline levels ($p>0.05$, Wilcoxon signed rank test). (B) At 4 weeks post-transplantation, C3a concentration in anti-CD40 Ab-treated primates (n=5) was significantly lower compared with that in rejected controls (n=4; *$p=0.0159$, Mann-Whitney U test). C3c deposits were barely observed in the graft with anti-CD40 Ab-treated primate (C), while extensive C3c depositions were observed in the graft of the rejected control (D; X200).

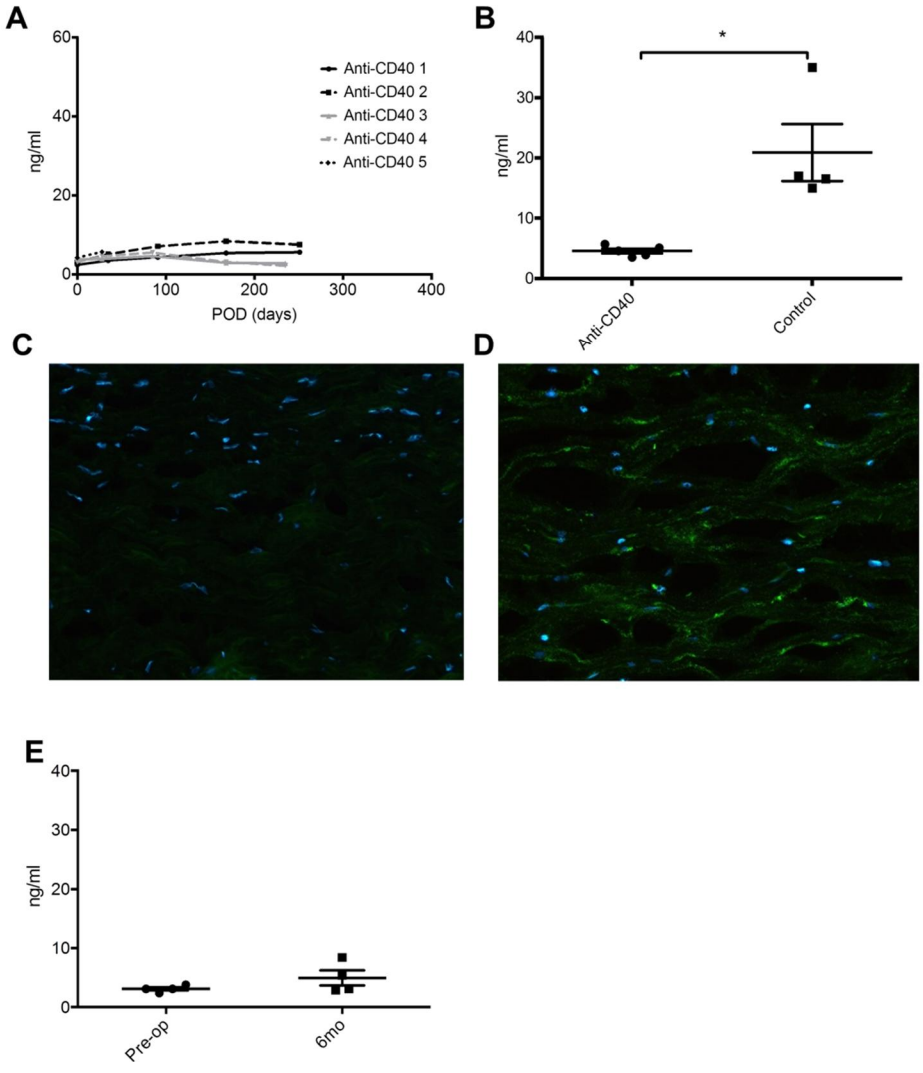

A
60
40
20
0
ng/ml
0
100
200
300
400
POD (days)
Anti-CD40 1
Anti-CD40 2
Anti-CD40 3
Anti-CD40 4
Anti-CD40 5
B
40
30
20
10
0
ng/ml
*
Anti-CD40
Control
C
D
E
40
30
20
10
0
ng/ml
Pre-op
6mo

Figure 11. Memory T cell changes in blood of anti-CD40 and anti-CD154 Ab-treated primates after transplantation.

(A-F) The changes of IFNγ+CD4+ or IFNγ+CD8+ T cells, effector (CD28-CD95+) memory CD4+ and CD8+ T cells, or central (CD28+CD95+) memory CD4+ and CD8+ T cells (black line) were not higher than those of preoperative states throughout the follow-up (baseline vs 6 months; $p>0.05$, Wilcoxon signed rank test; n=4) (G-K) The level of IFNγ+CD4+ or IFNγ+CD8+ T cells, effector/central memory CD4+ and effector memory CD8+ T cells were not different between anti-CD40 treated and anti-CD154 treated primates at baseline, at 1 month, and at 6 months after transplantation ($p>0.05$, Mann-Whitney U test). (L) The level of CD8+ central memory T cells was significantly lower in anti-CD40 treated primates at 1 month after transplantation ($p=0.0317$, Mann-Whitney U test). CM indicates central memory and EM indicates effector memory.

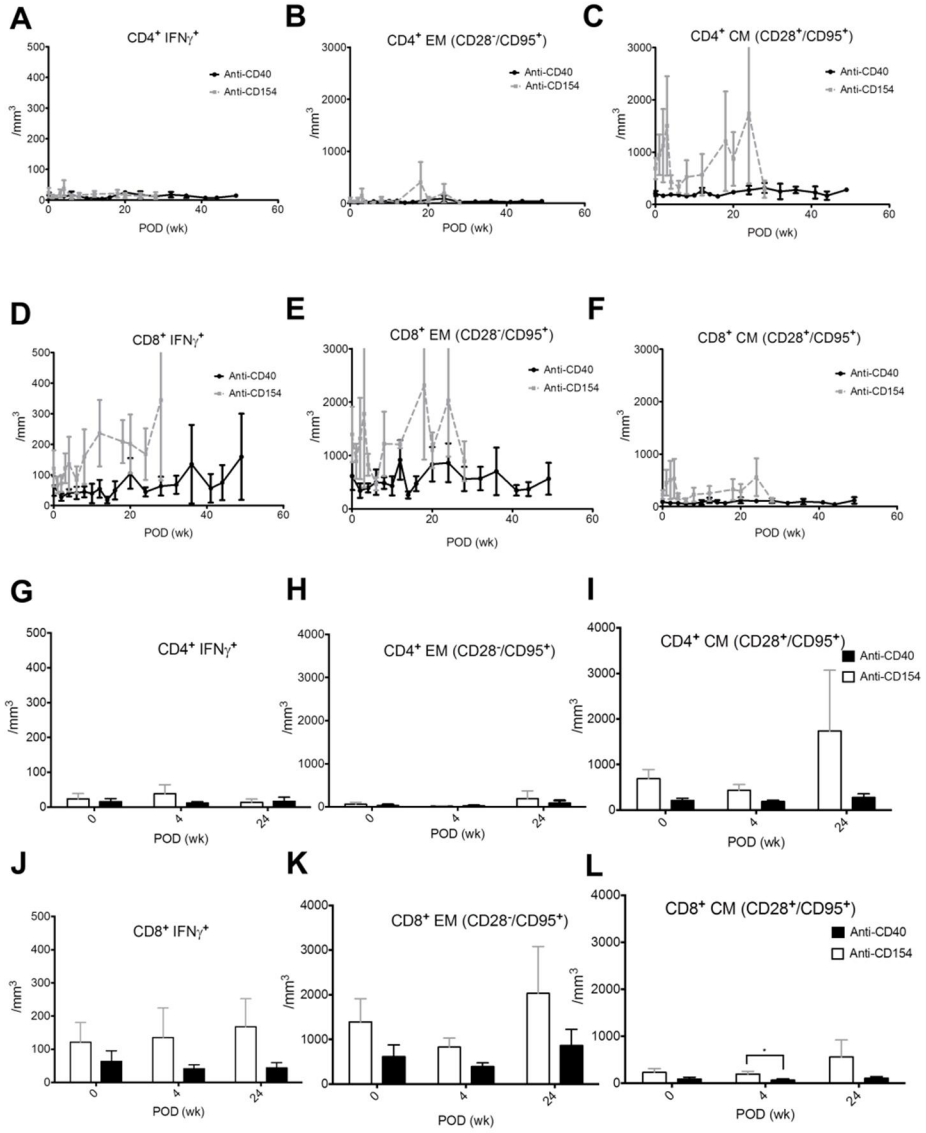
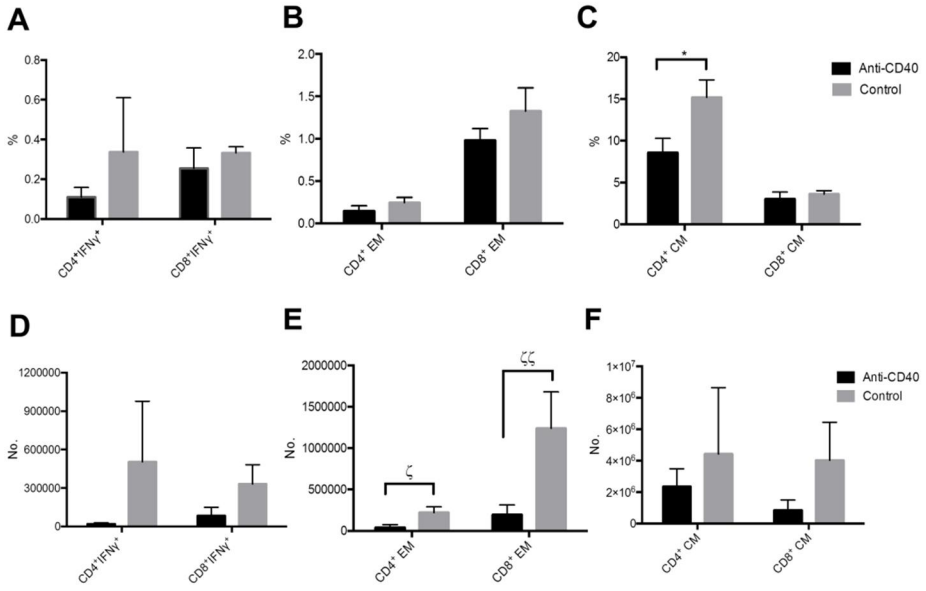

A
CD4+ IFNγ+
B
CD4+ EM (CD28-/CD95+)
C
CD4+ CM (CD28+/CD95+)
D
CD8+ IFNγ+
E
CD8+ EM (CD28-/CD95+)
F
CD8+ CM (CD28+/CD95+)
G
CD4+ IFNγ+
H
CD4+ EM (CD28-/CD95+)
I
CD4+ CM (CD28+/CD95+)
J
CD8+ IFNγ+
K
CD8+ EM (CD28-/CD95+)
L
CD8+ CM (CD28+/CD95+)
Anti-CD40
Anti-CD154
/mm3
POD (wk)

Figure 12. Comparative analysis of memory T cell changes in draining lymph nodes between anti-CD40 Ab-treated primates and rejected controls.

The mean percentage of central memory (CD28+CD95+) CD4+ T cells was lower in anti-CD40 Ab-treated primates (n=4) than in rejected controls (n=2) with a marginal significance (*p=0.082, Mann-Whitney U test; C). The number of effector memory (CD28-CD95+) CD4+ and CD8+ T cells were significantly lower in anti-CD40 Ab-treated primates than in rejected controls ($^{\zeta}$p=0.055, $^{\zeta\zeta}$p=0.032, Mann-Whitney U test; E). The changes of IFNγ+ CD4+ T cells were not significant. All data were analyzed after sacrifice (Anti-CD40 treated primates > 6 months, rejected control > 1month)


A
B
C
D
E
F
%
No.
0.8
0.6
0.4
0.2
0.0
2.0
1.5
1.0
0.5
20
15
10
5
1200000
900000
600000
300000
2000000
1500000
1000000
500000
1×10^7
8×10^6
6×10^6
4×10^6
2×10^6
*
ζ
ζζ
Anti-CD40
Control
CD4+IFNγ+
CD8+IFNγ+
CD4+ EM
CD8+ EM
CD4+ CM
CD8+ CM

DISCUSSION

Our data indicate that anti-CD40 monoclonal antibody (2C10R4) mediated-costimulation blockade effectively suppresses xenograft-related immune reaction and promotes long-term survival of deep-lamellar porcine corneal grafts in rhesus monkeys. Blockade of CD40/CD154 costimulatory pathway has emerged as a promising therapeutic modality in allotransplantation, xenotransplantation as well as autoimmune disease models.[15, 27-29] Although the treatment with anti-CD154 Ab has proven effective in xenotransplantation,[30,31] anti-CD154 Ab has yielded to antagonistic anti-CD40 Ab in the field of transplantation because of the thrombotic complications.[12] Despite a prior demonstration of the comparable xenograft survival outcome following the use of anti-CD154 Ab and anti-CD40 Ab,[29] efficacy of anti-CD40 Ab-mediated blockage in various xenotransplantation model has not been clearly established. Compared with the survivals of cellular lamellar keratoplasty using steroids in the other reports (21days-398days),[10] the survival of the cellular lamellar graft with anti-CD40 ab treatment seems to be pretty effective on corneal xenotransplantation. Most of the study achieved 0

to 60% of the 6 months-survival, while our study achieved 80% of the 6 months-survival.

The expression of CD40 or CD154 (CD40L) varies depending on the cells.[14,15,32] CD40 is constitutively expressed on B cells, macrophages, dendritic cells (DCs), monocytes, follicular dendritic cells, eosinophils and platelets, as well as on non-hematopoietic cells such as myofibroblasts, fibroblasts, epithelial, and endothelial cells. In addition, CD40 is transiently expressed on activated CD8+ T cells. Meanwhile, CD154 inducibly expresses mainly on activated CD4+ T cells, activated B cells, activated CD8+ and γδ T-cells, and platelets. Under inflammatory conditions, CD154 is also transiently induced on monocytes, natural killer cells, mast cells, and basophils and endothelial cells following activation as well as non-hematopoietic cells including epithelial and vascular endothelial cells, and smooth muscle cells. Therefore, anti-CD40 Ab-mediated blockage appears to have no direct effect on the activated CD4+ T cells unlike the anti-CD154 Ab-mediated blockage, presumably resulting in different efficacy on xeno-transplantation model.

From our data, anti-CD40 Ab treatment combined with IVIG showed effective down-regulation of donor-specific Ab responses, anti-αGal, as well as anti-non αGal Ab responses, complement changes and the responses of effector memory T cells, resulting in excellent survival of xenografts. We found initial peak of IgG responses which did not correlate with clinical progress. When we measured *in vivo* IgG reactivity against non-donor third party PBMCs using plasma from the primates given anti-CD40 Abs and IVIG (n=5), a similar spike was observed in early post-operative period (Figure 8A). The increased Ig G binding against porcine PBMCs was also observed when mixed with plasma of a naïve primate *in vitro* after addition of IV IG (Figure 8B). Plasma levels of anti-αGal IgG Abs in a naïve primate or a human were also increased in a dose-dependent manner after *in vitro* application of IVIGs (Figure 8C). In addition, a certain amount of anti-αGal IgG exists in IVIG agents. Taken together, these findings suggest that initial high peak of donor-specific Ig G or anti-αGal level is presumably caused by administration of IVIG.

The level of IFNγ+CD4+/ IFNγ+CD8+ T cells, effector memory and central memory T cells in the PBMCs were not higher than those between baseline and 6 months. It can be assumed that down-regulated dendritic cell activation by blockage of anti-CD40 Abs may contribute to reduced activation of CD4+ T cells. Considering that deep anterior lamellar keratoplasty without endothelial transplantation shows lower antigenicity than the full thickness keratoplasty, direct comparison of the efficacy between the anti-CD154 Ab and the anti-CD40 Ab is not possible in different keratoplasty settings. We compared the changes of T cells between the groups in order to use the data of anti-CD154 treated group as a reference level (negative control) to clarify the range that is tolerable for the T cell changes in successful survival. Compared to previous study with anti-CD154 Ab-treated primates, in which graft survival was excellent,[11] no significant changes in effector or central memory T cells were found between anti-CD40 and anti-CD154 Ab treatment, except for changes in CD8+ central memory T cells, which were lower in anti-CD40 abs-treated primates. This indicates that all of the changes in effector and memory T cells in PBMCs appear to be within clinically tolerable ranges,

which corresponds well with other clinical data. In addition, the fact that the numbers of effector memory CD4+ and CD8+ T cells were significantly lower in the lymph nodes of anti-CD40 Ab-treated primates than in rejected controls suggests the effective control of the effector memory T cell population by anti-CD40 Abs. We used mouse-rhesus chimeric form of 2C10R4 (IgG4 isotype) as antagonistic anti-CD40 Abs.

This anti-CD40 Ab-mediated inhibition works well *in vitro* and as corroborated by our *in vivo* models.[33] In our study, high dosage of anti-CD40 (50mg/kg) was initially administered for 8 times, and subsequently it was switched to low dosage administration (30mg/kg) for 7 times during 6 months, which worked efficiently. Although, human and rhesus CD40 share 95% homology of amino acid molecules, an *in vitro* study revealed lower inhibitory action of anti-CD40 Ab (clone of 2C10R4) against proliferation of human PBMCs compared with that against the proliferation of rhesus PBMCs.[33] Therefore, adjustment of the administration may be required in human clinical trials in dosages and frequencies to use anti-CD40 Abs from

the IgG4 isotype clone, or for human usage, different isotype IgG should be selected to generate anti-CD40 Abs.

One of the advantages of anti-CD40 from 2C10 appears to induce minimal depletion of B cells, although it effectively inhibited donor-specific and anti-α Gal responses. This property would be important to defense against viral infection under immune suppression after transplantation. In fact, our data (Supplementary Figure S2) revealed no clinically relevant activation of cytomegalovirus (CMV) infection. The antibody titers were subsequently increased which corresponded with the increase of the viral titer, resulting in no clinical activation of CMV. In addition, there were no considerable weight loss, fever, alanine aminotransferase changes, and blood urea nitrogen/creatinine changes in the anti-CD40 treatment (Supplementary Figure S3). All recipients showed weight loss during early postoperative period (average 10.2% loss from the initial weight for 2 weeks; 12.7%, 15.4%, 6.4%, 9.9, and 6.5%), however, they recovered the weight back to normal after 2 weeks of transplantation.

Notably, we found no expression of αGal in surviving graft; by contrast, abundant expression of αGal was found in rejected grafts which corresponds with our previous studies.[11] Although we do not know the exact reason why rejected grafts or grafts in the early post-operative period express αGal abundantly, it can be presumed that damaged or necrotic cells may expose more αGal on the cell surface due to conformational changes that are induced by attack from donor-specific immune cells or surgically induced inflammation. We presume that the reason why surviving grafts do not show any expression of αGals is because donor keratocytes may be slowly replaced in the anterior stromal grafts that have survived at least 6 months by recipient keratocytes from peripheral host tissues.

In conclusion, anti-CD40 Ab-mediated costimulatory blockage works well in deep anterior lamellar keratoplasty in NHPs. The data are a necessary and promising prelude to clinical trials in xenocorneal transplantation. Currently, we are investigating the efficacy of anti-CD40-mediated blockage in full thickness xenocorneal transplantation using SNU miniature WT pigs in NHPs

based on this study. We think the porcine cornea might be a promising substitute for the human cornea with relevant immunosuppression in the near future.

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초 록

목적: 이종각막이식은 인간 공여각막의 부족 문제를 해결할 수 있는 효과적인 방안이며, 돼지 각막은 광학적 생리학적 성질이 인간 각막과 유사하여 이종각막이식의 적절한 공여조직이라 할 수 있다. 그러나 이종각막이식에서는 이종간 항원의 차이를 극복하기 위해 강력한 면역억제제의 사용이 필요하다. 본 연구에서는 이종각막이식의 임상적용을 큰 목표로 돼지-영장류간 심부표층각막이식 모델에서 항 CD40 항체를 통한 공자극 차단 면역억제법의 효과를 알아보고자 하였다.

방법: 다섯 마리의 영장류(Chinese rhesus macaques)에서 7.5mm 직경의 돼지 각막을 이용하여 심부표층각막이식을 시행하였다. 면역억제를 위해 항 CD40 항체를 정해진 계획에 맞추어 정맥내로 투여하였다. 각막편 생존여부, 중심 각막 두께, 안압을 정기적으로 측정하였고, 영장류의 혈액 내 작동 및 기억 T 세포, 항 αGal 항체, 공여자 특이 항체, 방수 내 보체의 변화 등을 조사하였다. 항 CD40 항체 치료군에서의 기억 T 세포 특성을 이전 연구에서의 항 CD154 항체 치료군 또는 거부반응이 생겼던 대조군에서 기억 T 세포 특성과 비교하였다. 또한 anti CD40 항체 치료군에서 이식

전과 이식 6 개월 후 시점에서의 항 αGal, 항 non-αGal, 공여자 특이 항체, T 세포 분포를 비교하였다.

결과: 항 CD40항체를 이용한 공자극 차단은 이종각막편의 생존을 효과적으로 유지시켰으며(389, 382, 236, 201, 61일 이상), 80%에서 6개월 이상의 각막편 생존을 보여주었다. 항 CD40항체 투여군에서 이식 거부반응 대조군에 비해 유의하게 각막편의 염증세포의 침윤이 줄었고, 방수내 C3a 농도가 감소되었다. ($p=0.0159$, Mann-Whitney U test). 작동 기억 T 세포의 증가는 이식 거부반응 대조군에 비해 항 CD40 항체 치료군에서 효과적으로 억제되었다. ($p<0.05$, Mann-Whitney U test) 항 CD40항체 투여군에서 항 αGal, 항 non-αGal, 공여자 특이 항체는 이식 6개월후에도 이식 전 상태에 비해 유의하게 증가하지 않았다. ($p>0.05$, Wilcoxon signed rank test).

결론: 항 CD40 항체를 이용한 공자극 차단은 돼지-영장류 간 이종 심부표층각막이식에서 효과적인 면역억제법이 될 수 있다. 향후

이종각막이식의 임상 적용시, 항 CD40 항체를 유용한 면역억제제로 사용해 볼 수 있겠다.

주요어: 항 CD40 항체, 심부표층각막이식, 영장류, 이종이식

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